

TUMORIGENICITY AND LYSIS BY NATURAL KILLERS*

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We have previously described (1) the *in vitro* isolation of chemically transformed cell lines resistant (C type) and sensitive (I type) to rejection mechanisms operative in normal mice. The experiments reported here utilize these two classes of transformed fibroblastic cell lines (I and C type) along with the parental nontransformed cell line (N type) to analyze the resistance or sensitivity of these cell types to natural killer (NK)¹-mediated lysis and the specificity of NK cell recognition.

NK is a term used to describe the lytic activity of spleen cells from naive mice. The ability of NK cells to recognize and to lyse neoplastic but not normal cells has suggested the hypothesis that they are involved in tumor surveillance (2-4). This preferential lysis by NK cells has been interpreted to mean that NK-sensitive target cells express surface determinants recognized by NK cells, whereas normal or neoplastic resistant target cells do not express these determinants. The experiments of Roder et al. (5), using lymphoid cell lines, support this hypothesis. They isolated "NK target structures" and have shown that these molecules are found on NK-sensitive cells and are absent on NK-resistant cells whether normal or neoplastic. Here, we show that this conclusion is not valid for all NK targets.

Fibroblasts whether normal or neoplastic, which are not lysed by NK cells, are nevertheless recognized by, but resistant to the NK effector mechanism. If NK is involved in surveillance of transformed cells, then the presence of NK target structures on NK-resistant cells must limit the effectiveness of surveillance for all cell types including lymphoid. This finding has as its consequences the following: (a) for some cell types the difference between resistance and sensitivity is not caused by recognition but rather by a subsequent step on the pathway to lysis; (b) there are either two types of NK lytic signals, one for lymphoid cells and another for fibroblasts, or the two classes of target cells react differently to the same lytic signal; and (c) if NK activity plays a significant role in tumor surveillance, a special mechanism must operate to enable the NK effector to find efficiently and lyse the transformed target.

Materials and Methods

Mice. BALB/c mice were from the Salk Institute colony. Immune-deprived mice were prepared by thymectomy of 4- to 6-wk-old mice followed 2 wk later by lethal irradiation (750 rads) and reconstituted with $1-2 \times 10^7$ syngeneic fetal liver cells (ATxFL).

Cell Lines. The cell lines are classified as N-, I-, or C-type cells according to the following characteristics:

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¹ Abbreviations used in this paper: ATxFL, an adult thymectomized, lethally irradiated, fetal liver reconstituted mouse; FBS, fetal bovine serum; NK, natural killer cell-like.

Cell type	In vitro		In vivo tumorigenic potential in	
	Continuous cell line	Growth in agarose	ATxFL	Normal
Primary fibroblast	-	-	-	-
N type	+	-	-	-
I type	+	+	+	-
C type	+	+	+	+

The cells studied here were derived as lineages (see top of Figs. 1 and 2), and were maintained in Dulbecco's modified Eagle's media plus 10% fetal bovine serum (FBS). B/C-N is a cloned fibroblastic cell line derived by us from BALB/c fetal tissue. It is classified as an N-type cell line. The fibroblast population from which it was derived is referred to as primary fibroblasts.

10ME HD A.5 R.1 (10ME) is a cloned transformed cell line derived from B/C-N after treatment with 3-methylcholanthrene-11,12-dihydroepoxide (supplied by National Cancer Institute Carcinogenesis Research Program). This transformed variant was selected by growth in 0.3% agarose then ring cloned, and is classified as an I-type cell line.

10CR A.2 R.1 (10CR) is a cloned cell line derived from B/C-N after growth in the presence

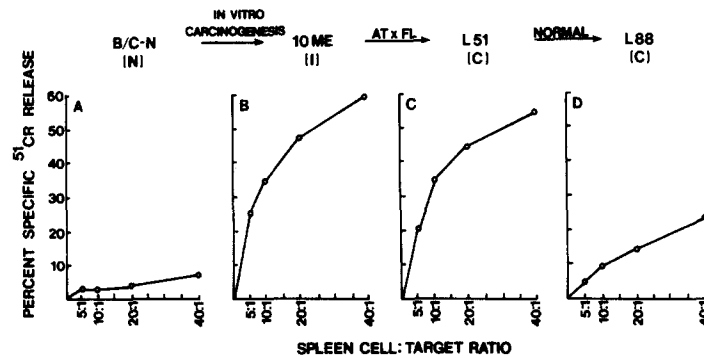


FIG. 1. Titration of normal spleen cell NK activity directed against N-, I-, and C-type target cells. The targets are B/C-N (A), 10ME (B), L51 (C), and L88 (D). These targets were derived sequentially as indicated at the top of the figure, and the classification (N, I, or C) is indicated in the parenthesis under each cell line. For more details, see Materials and Methods.

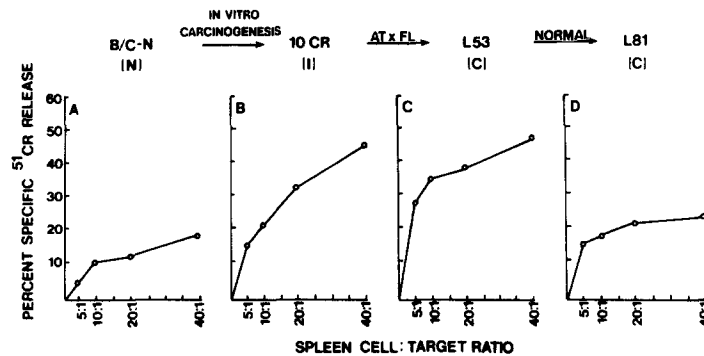


FIG. 2. Titration of normal spleen cell NK activity directed against N-, I-, and C-type target cells. The targets are B/C-N (A), 10CR (B), L53 (C), and L81 (D). These targets were derived sequentially as indicated at the top of the figure, and the classification (N, I, or C) is indicated in the parenthesis under each cell line. For more details, see Materials and Methods.

of methylcholanthrene crystals. This transformed variant was selected by growth in 0.3% agarose then ring cloned, and is classified as an I-type cell line.

L50 and L51 are uncloned cell populations established from the tumors that arose in ATxFL mice after the subcutaneous injection of 10^7 10ME cells. These cell lines are classified as C type.

L53 is an uncloned cell line established from a tumor in an ATxFL mouse after the subcutaneous injection of 10^7 10CR cells. This cell line is classified as C type.

L81 and L88 are uncloned cell populations established from tumors in normal mice after the subcutaneous injection of L53 and L51, respectively, and are classified as C type.

Chromium-release Assay for NK-mediated Lysis. Spleens were dispersed into single cells and suspended in RPMI-1640 media plus 10% FBS supplemented as previously described (6). ^{51}Cr -labeled targets were prepared as previously described (7). When unlabeled targets were used (i.e., competitors), they were mixed with labeled targets before their addition to assay vessels containing spleen cells. Assays done in microtiter plates contained 10^4 ^{51}Cr -labeled targets per well in 100- μl media; whereas assays done in 35-mm dishes utilized 5×10^4 ^{51}Cr -labeled targets per dish. The assay vessels were gently rocked for approximately 15 h at 37°C , then centrifuged at 160 g for 5 min, and half of the supernate was collected to determine the amount of ^{51}Cr released.

The percent specific ^{51}Cr released was calculated by the following formula: % specific ^{51}Cr release = (sample counts per minute - spontaneous counts per minute)/(total counts per minute - spontaneous counts per minute). All data points represent the arithmetic mean of triplicate samples. In all assays the spontaneous release of ^{51}Cr ranged from 16 to 32%. When protein synthesis inhibitors were added to the reaction, the spontaneous release was determined in the presence of these inhibitors and was slightly higher than in their absence (see Fig. 12).

Relative sensitivity to NK lysis of different targets can be assessed by comparing the number of effector cells required to give an equivalent amount of chromium release, or by comparing the amount of chromium released at any one effector concentration.

Results

Lysis of I-type Targets is Caused by NK Activity. Because I-type cell lines grow as tumors in ATxFL mice, yet are rejected by normal mice, we initiated a series of experiments to determine if T cell-mediated cytotoxicity, which is absent in ATxFL mice, was responsible for the rejection of the I lines in normal mice. To do this we examined the in vitro lysis of N, I, and C cells by spleen cells from mice primed in vivo and boosted in vitro with appropriate N, I, or C cells. In summary, these experiments showed that there was no inducible cell-mediated lysis of the targets over the lytic activity of the nonimmunized, control spleen cells. The failure to induce a cell-mediated response and the high level of killing observed in vitro by spleen cells from naive mice suggested that NK-like activity was responsible for the cytotoxicity in these experiments. We confirmed this by the following four criteria:

(a) The lysis of the target cells is not dependent on immunoglobulin (Ig)-bearing cells because the amount of lysis is not diminished by passing spleen cells through an anti-Ig column (8) or by the direct addition of purified anti- κ light-chain antibody, which inhibits antibody-dependent cellular cytotoxicity, to the chromium-release assay (9).

(b) The spleen cell activity is not reduced by removal of adherent cells or elimination of T cells with anti-Thy 1.2 plus complement or elimination of B cells by treatment with anti-Ig plus complement (10).

(c) The lytic activity of spleen cells is reduced if mice are pretreated with hydrocortisone, which is known to reduce NK activity (11).

(d) The lytic activity of spleen cells is not H-2 restricted, which rules out cytotoxic T cells as the effectors. It was also observed that the activity of spleen cells is not

increased after tumor inoculation. We found no evidence for lytic activity that was inducible, NK or other.

N Lines, Unlike I Lines, are Resistant to NK Lysis. The next series of experiments were designed to see if there was a correlation between the level of sensitivity to NK lysis and tumorigenicity by comparing the NK sensitivity of the N-type parental cell line, B/C-N, and two I-type cell lines derived from it (Figs. 1 and 2). If NK activity were responsible for the rejection of I cell lines, then the I-type lines would be sensitive to NK lysis. By this same reasoning, the C type-transformed cells would be resistant to NK lysis because these cell lines grow as tumors in mice with normal NK activity. Because the nontransformed N-type cell lines do not form tumors in any test mice, we would also expect these cells to be resistant to NK lysis, along with all other normal cells. In fact, we found the N-type cell line (B/C-N) to be relatively resistant to NK lysis, whereas both I-type lines (10ME and 10CR) were found to be extremely sensitive. By a direct comparison of the percent specific ^{51}Cr release (at a given spleen cell:target ratio), the I-type lines are approximately fourfold more sensitive than the N-type line. By comparing the spleen cell:target ratio (at a given percent specific ^{51}Cr release), I-type cells are ~16-fold more sensitive than the N-type cell line.

ATxFL, Unlike Normal Mice, are Deficient in NK Activity. Because I-type cell lines are not rejected by ATxFL mice, we tested the in vitro NK lytic activity of ATxFL mouse spleens. As shown in Fig. 3, ATxFL mouse spleen cells tested on the most sensitive I line (10ME) are very low in NK activity 5 wk after irradiation and fetal liver restoration. It has been shown that NK activity is radiosensitive, but gradually returns (12) to near normal levels after 8 wk. Similar results were observed for the ATxFL mice. The results of these experiments are consistent with the hypothesis that I-type cell lines form tumors in ATxFL mice because these animals have low levels of NK activity, but do not form tumors in normal mice because of normal levels of NK activity.

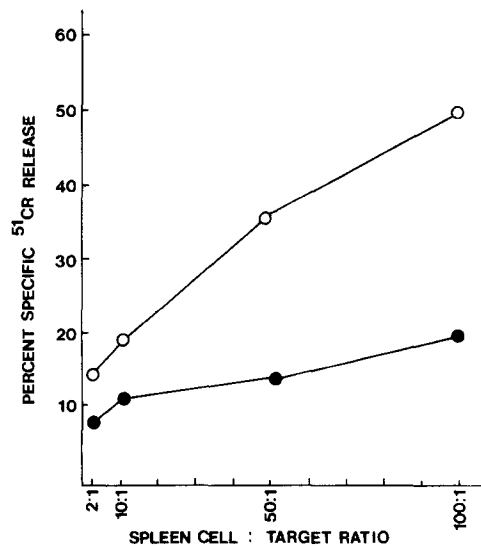


FIG. 3. Comparison of the NK activity of spleen cells from normal mice (○) and ATxFL mice 5 wk after irradiation and restoration (●). The target is 10ME.

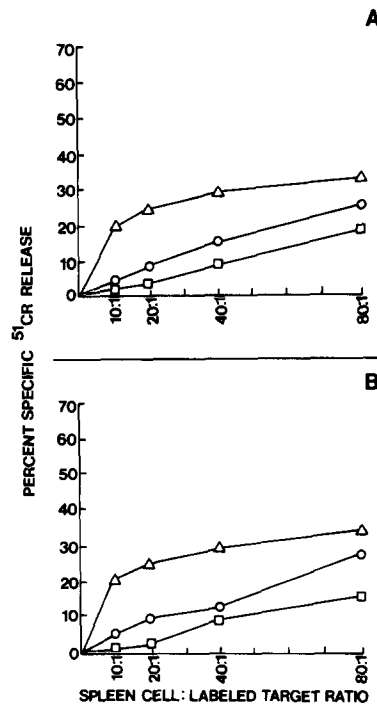


FIG. 4. Competition for the NK lysis of 10CR by 10CR (A) and 10ME (B). The ratio of unlabeled targets to labeled targets was 0:1 (Δ), 1:1 (\circ), and 3:1 (\square).

C-type Mutants Derived from I-type Cells By Sequential Passage in ATxFL Then Normal Mice are Resistant to NK Lysis. The cell lines, L51 and L53, were established from tumors that arose in ATxFL mice after the injection of the I-type cell lines, 10ME and 10CR, respectively. These tumor cell lines were found to be capable of forming tumors in both ATxFL and normal mice in spite of the fact that their parental cell lines, 10ME and 10CR, will only form tumors in ATxFL mice.

The comparison of the tumorigenic potential of L51 and L53 (C type) with that of 10ME and 10CR (I type) indicates that selection for increased tumorigenicity has occurred in the ATxFL mice. If the selective pressure in ATxFL mice is NK activity then it must operate late in tumor growth because these animals are devoid of NK activity at the time when cells are injected, and it must be weak because the uncloned tumor cell lines (L51 and L53) are NK sensitive like their parental I-type cell lines (see Figs. 1 C and 2 C). If these populations (L51 and L53) are mixtures of sensitive and resistant cells, up to 20% of the cells could be resistant and go undetected in our *in vitro* NK assay.

The cell lines, L88 and L81, were established without cloning from the passage of L51 and L53 in normal mice. The lines, L88 and L81, are NK resistant (see Figs. 1 D and 2 D). From these results it is clear that the ability of a transformed cell to grow as a tumor in normal mice is correlated with resistance to NK-mediated lysis.

There remain the questions as to why I-type lines are so highly sensitive to NK lysis and how N- and C-type cells resist lysis. To answer these questions we were led to a

series of experiments aimed at analyzing the NK recognition determinants on these sensitive and resistant fibroblast lines.

NK-resistant Cells Compete for NK Lysis of Sensitive Cells. Fig. 4 shows the NK lysis of a ^{51}Cr -labeled I-type cell line, 10CR, and the competition for the killing by the autologous I-type line as well as another independently derived I-type cell line, 10ME. A comparison of these two curves shows that both cell lines compete equally well. Thus, any determinants recognized by NK cells on 10CR targets are also expressed on 10ME cells. However, there may be determinants present on 10ME that are not expressed on 10CR. In the converse experiment using ^{51}Cr -labeled 10ME target cells with unlabeled 10CR and 10ME cells as the competitors (Fig. 5) a similar result was obtained, i.e., both cell lines compete equally well. These reciprocal experiments indicate that two independently derived chemically transformed clones that are sensitive to NK lysis, are recognized identically by NK effector cells. Further experiments using the C-type cell line L88 to compete for the NK lysis of 10ME showed

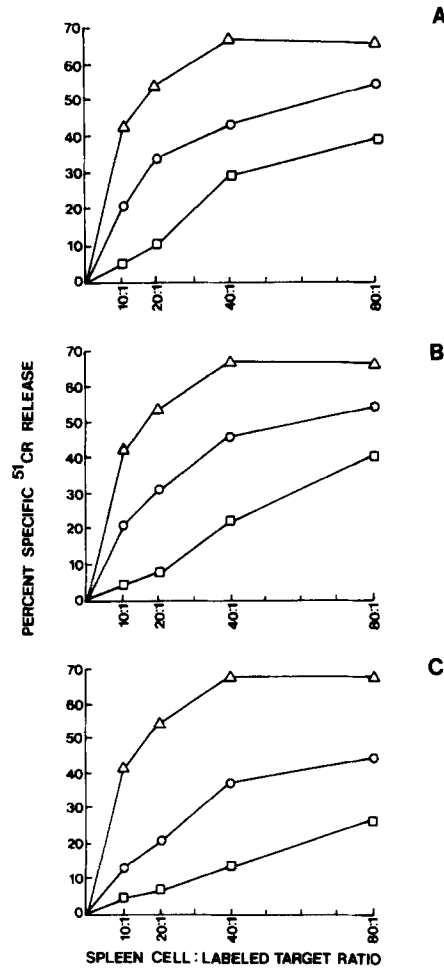


FIG. 5. Competition for the NK lysis of 10ME by 10ME (A), 10CR (B), and B/C-N (C). The ratio of unlabeled targets to labeled targets was 0:1 (Δ), 1:1 (\circ), and 3:1 (\square).

that this C-type cell line, which is resistant to NK lysis, is also a good competitor (see Fig. 6), and, therefore, must also express the same NK recognition determinants expressed on 10ME and 10CR. Fig. 5C shows the competition for lysis of chromium-labeled 10ME by the N-type fibroblast, B/C-N. This cell line competes as well as any other competitor tested, a result that is of key importance because B/C-N is not a transformed cell and is not a NK-sensitive target.

In order to determine if cells outside of these lineages also express NK recognition determinants, competition experiments were performed using fresh normal tissue as well as independently derived cell lines characterized as neuronal and fibroblastic. All cells tested, except adult thymocytes (Fig. 7), WEHI 7, a T cell lymphoma, and the N18 derivative of C1300, a neuronal cell line (Table I) compete in NK lytic assays on NK-sensitive fibroblast targets. Because primary fetal fibroblasts, adult fibroblasts (Fig. 8), or fibroblastic cell lines BNL 2/5, L47, as well as neuronal and lymphoid cell lines (Table I and Fig. 9) act as competitors, the determinants recognized by NK cells are not unique to fibroblast targets.

It is conceivable that some of the competition might be a result of steric hindrance of NK effectors in the assay vessel when the number of target cells is increased by the addition of competitor cells. The lack of competition by thymocytes (Fig. 7) and C1300-N18 (Table I) indicates that this is not the situation. The failure of thymocytes to compete is not related to their lack of adherence to plastic because another nonadherent lymphoid cell line, YAC-1 (an NK-sensitive target), is a good competitor (see Fig. 9). In addition, because N18 is an adherent cell line, competition is not a result of attachment of competitors to the assay vessels. The lack of competition by normal thymocytes cannot simply be caused by the difference in size between fibroblasts and thymocytes because the level of competition by thymocytes at 20 unlabeled competitors to 1 labeled target is, at best, equivalent to the level of competition of only 1 unlabeled fibroblast to 1 labeled target. We have performed these assays in both microtiter plates and 35-mm dishes with comparable results. In

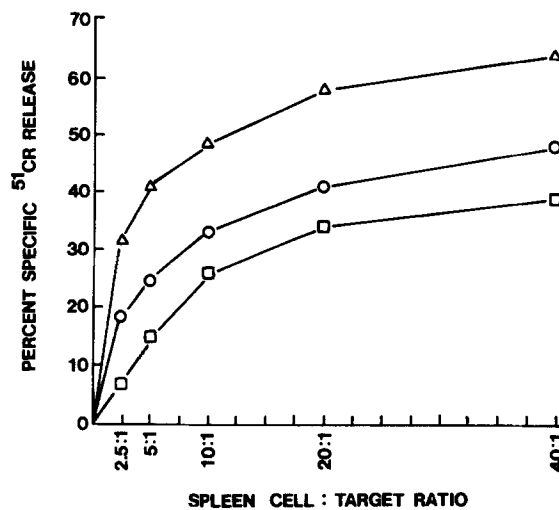


FIG. 6. Competition for the NK lysis of 10ME by L88. The ratio of unlabeled targets to labeled targets was 0:1 (Δ), 1:1 (○), and 3:1 (□).

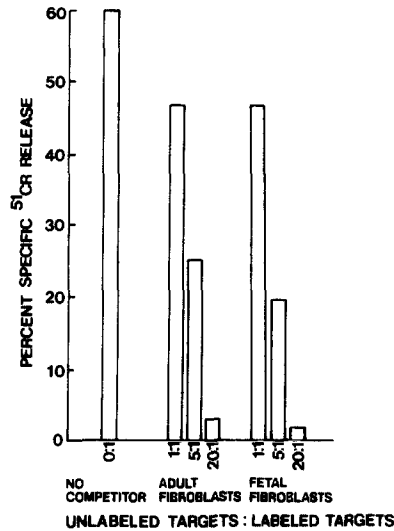


FIG. 7. Competition of the NK lysis of 10ME by primary adult and fetal fibroblast cultures. The ratio of labeled 10ME to NK effectors (spleen cells) was 1:100.

TABLE I
Competition for the Killing of an NK-sensitive Target (10ME) by Various Cell Types

Unlabeled target (one labeled 10ME/one unlabeled target)	Percent ⁵¹ Cr released at an effector: labeled target ratio of	
	20:1	10:1
Experiment 1		
None	89	78
10ME	62	38
BNL 2/5*	59	53
BNL SVA.8‡	50	45
Et R7§	32	22
C1300 N18	97	83
Experiment 2		
None	77	60
10ME ■	53	44
WEHI 7¶	73	63
WEHI 7 (unlabeled target:10ME:20:1)	71	63
L47**	43	34

* Cloned, nontumorigenic BALB/c fibroblastic cell line derived from mouse liver.

‡ Cloned cell line derived from BNL 2/5 transformed in vitro with SV40 virus.

§ Cloned neuronal cell line from a C57BL/6 mouse (15).

|| Cloned neuroblastoma cell line from a A/J mouse (16).

¶ Cloned T cell lymphoma from a BALB/c mouse.

** Cell line derived from a tumor in BALB/b mouse after the injection of an in vitro chemically transformed BALB/b fibroblast cell line.

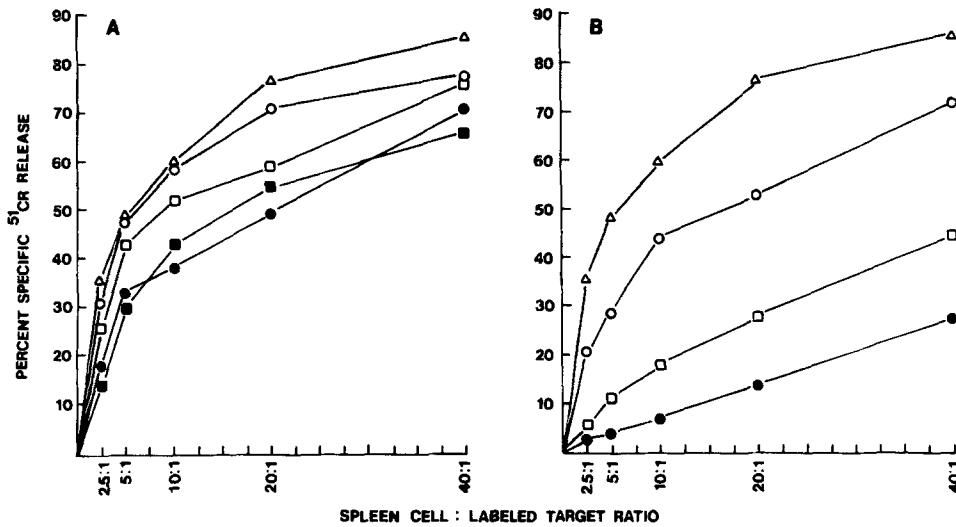


FIG. 8. Competition for the NK lysis of 10ME by normal thymocytes (A) and 10ME (B). The ratio of unlabeled targets to labeled targets was 0:1 (Δ), 1:1 (\circ), 3:1 (\square), 6:1 (\bullet), 20:1 (\blacksquare).

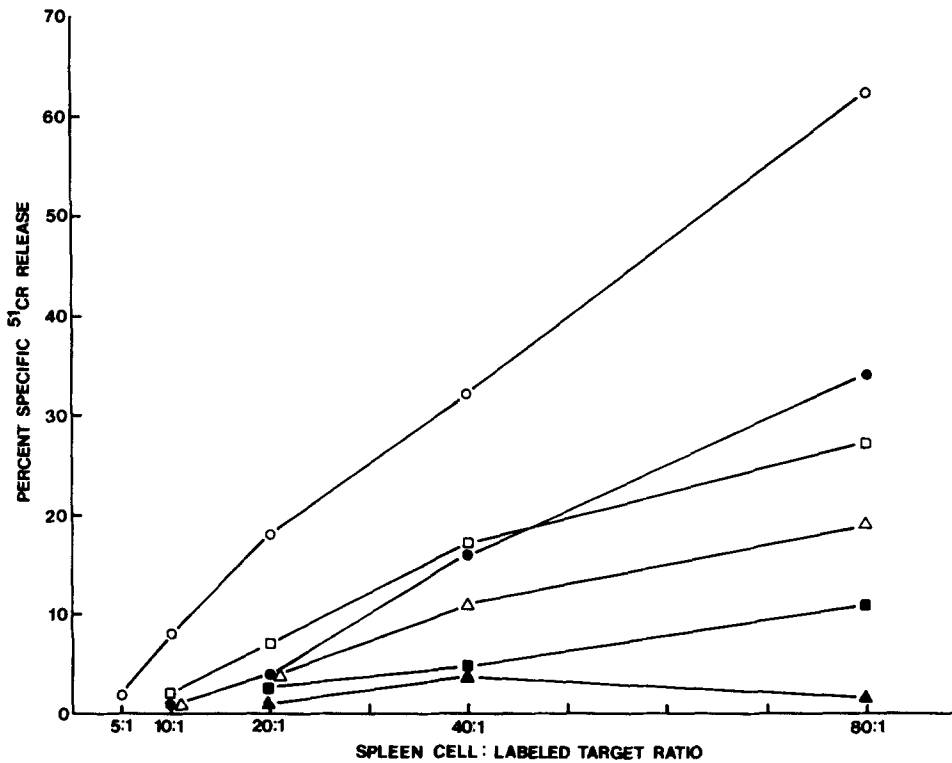


FIG. 9. Competition for the NK lysis of 10ME by 10ME and YAC-1. The ratio of unlabeled 10ME to labeled 10ME was 0:1 (\circ) and 3:1 (\square). The ratio of unlabeled YAC-1 to labeled 10ME was 1:1 (\bullet), 3:1 (Δ), 6:1 (\blacksquare), and 18:1 (\blacktriangle).

microtiter plates there were 500 targets/mm² whereas in small dishes there were only 50 targets/mm². Thus, there appears to be little effect caused by the density of the targets or spleen cells within the assay vessels, and, as an approximation, it is the ratio of targets (labeled and unlabeled) to spleen cells that determines the extent of chromium release. Furthermore, the fact that B/C-N is lysed in the presence of protein synthesis inhibitors (see below) indicates that competition by these cells is at the level of recognition.

NK Lysis of Fibroblast Targets Shows a Lag Period. The competition data imply that both sensitive and resistant cells possess the cell surface determinants recognized by NK effectors. Therefore, sensitivity or resistance must reside in later steps involving either induction and/or activation of the lytic mechanism or repair of the lytic damage (counterlytic activity).

The lysis of fibroblastic targets shows a 4- to 6-h lag after the mixing of the NK effector cells (spleen cells) with the ⁵¹Cr-labeled target cells (Fig. 10). After this lag period, the release of ⁵¹Cr proceeds at a relatively constant rate over the next 8-10 h. Because fibroblasts, but not lymphoid targets, show this lag period, we initiated a series of experiments to determine if the lag represents the time required for NK effectors to be activated by fibroblasts target cells.

The Lag is Not Caused by Activation of NK Effector Cells by Sensitive Targets. If lysis of target cells were dependent upon activation of NK effector cells, then the lag could represent the time required for the activation. To test this hypothesis, spleen cells were added to microtiter plates along with 10⁴ unlabeled 10ME, B/C-N, or no target cells. 4 h later (t = 4), 10⁴ chromium-labeled 10ME cells were added to all the plates. 1 h later (t = 5) and every 2 h thereafter (up to t = 19) supernates from each pretreatment group were harvested and the percent specific ⁵¹Cr release determined.

The results of this experiment are shown in Fig. 11. The failure of unlabeled B/C-N or 10ME added at t = 0 to alter the lag time of ⁵¹Cr release from labeled 10ME added at t = 4 indicates that in vitro activation of NK cells is not responsible for the lag period.

Resistance to Lysis is Dependent on Protein Synthesis, Whereas the Lag is Not. In order to

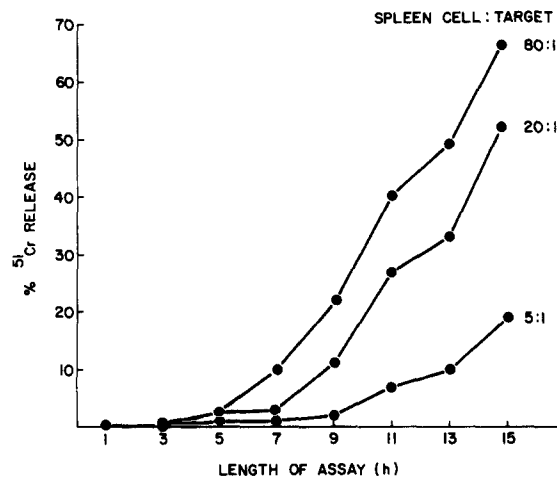


FIG. 10. Kinetics of chromium release assays of NK-mediated lysis of 10ME.

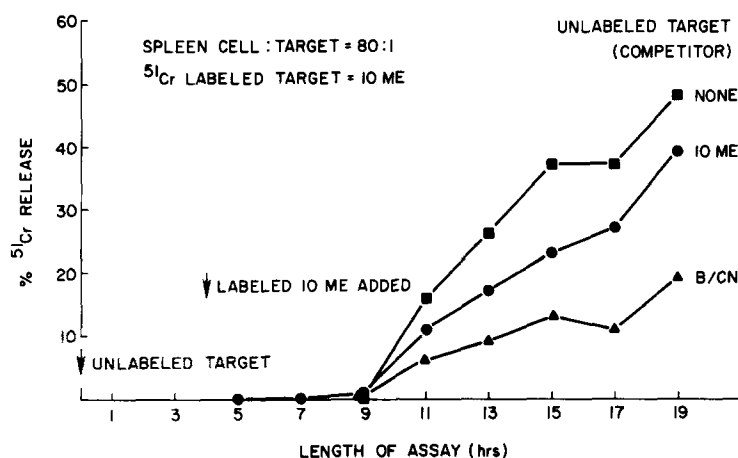
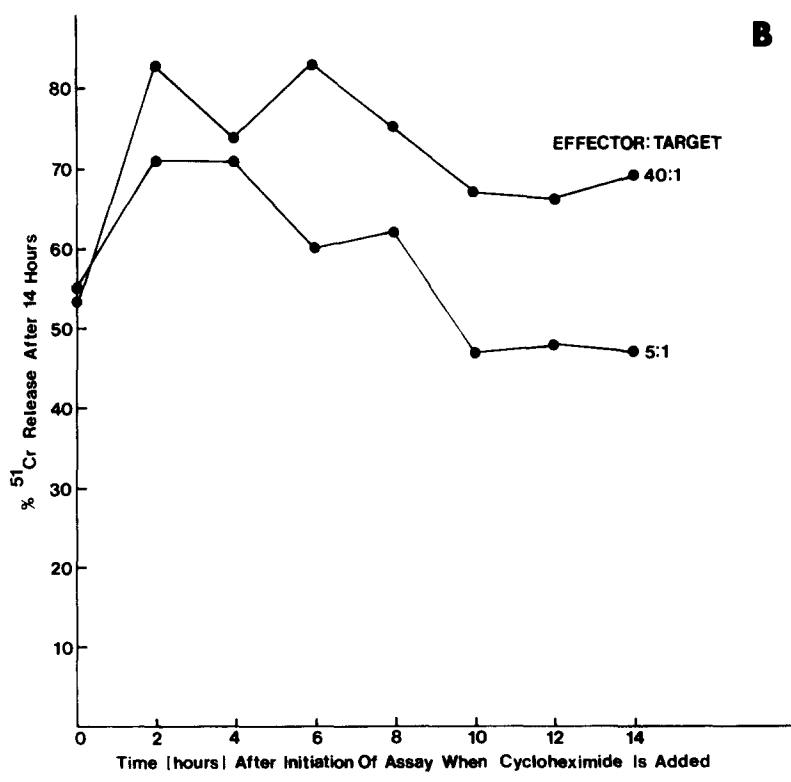
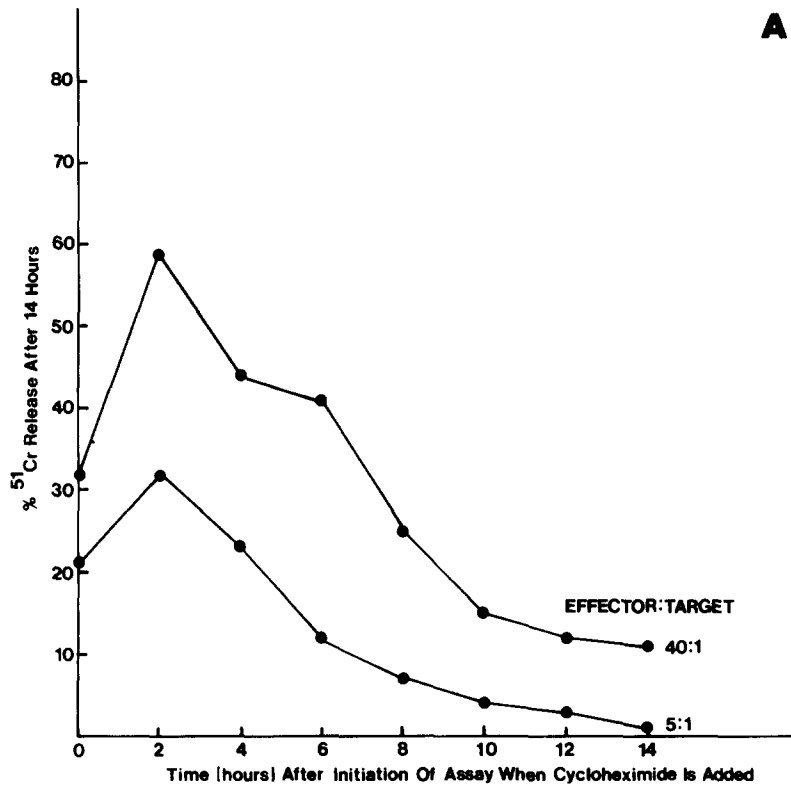


FIG. 11. Kinetics of chromium release assays of NK-mediated lysis of 10ME after preincubation of effectors with 10ME, B/C-N, or nothing.

test whether the lag was caused by a requirement for *de novo* protein synthesis of lytic enzymes by either the target or effector cell, we performed the NK assay in the presence of the protein synthesis inhibitor, cycloheximide. If protein synthesis is required for lysis, then there should be a decrease in the killing of sensitive or resistant target cells in the presence of cycloheximide. Although our experiments show that the lag period is not due to the requirement for *de novo* protein synthesis, these experiments do show that resistance to NK lysis is dependent on protein synthesis.

In these experiments two sets of NK assay plates were prepared; one with 10ME as the target cell and the other with B/C-N as the target cell. At the initiation of the assay, and at 2-h intervals thereafter, cycloheximide was added to one plate from each set (5 mM); regardless of when cycloheximide was added, all the assays were terminated at 14 h and the percent specific ⁵¹Cr release was determined. The results of these experiments are shown in Fig. 12. When the NK assay was run with no cycloheximide present (cycloheximide added at $t = 14$), 10ME was lysed (Fig. 12 B), whereas B/C-N was NK resistant (Fig. 12 A). When the assay was allowed to proceed for 8–12 h before the addition of cycloheximide (then 4–6 h in the presence of the inhibitor), there was little effect on the results. Surprisingly, when the assay was allowed to proceed for 0–6 h before the addition of cycloheximide (8–14 h with cycloheximide), an increase in target lysis was observed. This is most striking with B/C-N when the assay proceeds for 2 h without cycloheximide and then the remaining 12 h of the assay period with cycloheximide present (see Fig. 12 A). Here, the control level of NK-mediated killing (i.e., cycloheximide added at $t = 14$) was 11% (spleen cell:target ratio of 40:1), whereas the experimental level of NK lysis at the same spleen cell:target ratio, with cycloheximide added at $t = 2$, was 59%. Similar results have been observed with every NK-resistant fibroblast tested.

Cycloheximide blocks protein synthesis preventing both chain initiation and elongation by an interaction with the 60S ribosomal subunit (13). Two other inhibitors of protein synthesis had effects similar to cycloheximide when added to the NK assay. One of these, streptimidone, functions as does cycloheximide, whereas the other, emetine, functions at the level of the 80S ribosomal unit and not the 60S subunit (13),



further supporting our interpretation that the effect of cycloheximide in these experiments is actually at the level of protein synthesis.

These results lead to three important conclusions. First, the lag period is not caused by induction of lytic enzymes because the addition of protein synthesis inhibitors at the beginning of the assay does not reduce lysis. Second, B/C-N must bear NK recognition determinants because it is lysed by NK effectors when protein synthesis inhibitors are added at the appropriate times during the assay. This result strongly supports our interpretation that the competition experiments reflect the presence of NK target determinants on both resistant B/C-N and sensitive I-type cells (see Fig. 5). Third, B/C-N is NK resistant because of a counterlytic mechanism, the expression of which depends upon protein synthesis. The NK-sensitive targets (10ME and 10CR) must have a lower level of counterlytic activity than the NK-resistant target, B/C-N. These conclusions will be developed in the discussion.

Discussion

We have analyzed the NK sensitivity of cells at various stages on the pathway to cancer. The nontransformed parental N-type cell line, B/C-N, is anchorage dependent and nontumorigenic. I-type cell lines are anchorage independent and grow as tumors only in surveillance depressed mice (ATxFL). C-type cell lines are anchorage independent and grow as tumors in both ATxFL and normal mice. C-type cells must, therefore, escape a surveillance mechanism, which prevents I-type cells from growing as tumors in normal mice.

The observations that I-type lines are sensitive to NK-mediated lysis, whereas the nontransformed N-type and those transformed C-type lines derived as tumors from normal mice are resistant to NK-mediated lysis, are consistent with the hypothesis that escape from lysis by NK is obligatory for tumor formation in normal mice. The finding that those C-type cell lines derived from tumors formed by the injection of I-type lines into ATxFL mice are NK sensitive (e.g., L51, L53) is also consistent with this hypothesis because the tumors that develop in the normal mice as a result of the injection of either L51 or L53 are NK resistant (e.g., L81, L88).

This association between tumorigenicity and NK sensitivity, as predicted by a NK surveillance hypothesis, is made ambiguous by the lack of a correlation between binding specificity and either NK sensitivity or tumorigenicity. In addition to competition for NK lysis of one transformed cell line by other transformed cells, the nontransformed parental cell line B/C-N, as well as primary fibroblasts, neuronal, and lymphoid cell lines also compete, which indicates that the NK recognition determinant present on NK-sensitive cells is also present on a variety of cell types, both sensitive and resistant.

The fact that cycloheximide renders the normally resistant B/C-N sensitive to NK lysis confirms our interpretation of the competition studies showing that NK effectors recognize determinants present on both sensitive and resistant target cells. The effect

FIG. 12. Effects of the addition of cycloheximide on the chromium release assay of NK-mediated lysis of a resistant, B/C-N (A) and a sensitive, 10ME (B), target. The spontaneous release of chromium in the presence of cycloheximide (t equals the time of addition of the cycloheximide) from B/C-N (A) was 26% at t = 14, 27% at t = 12, 26% at t = 10, 26% at t = 8, 25% at t = 6, 24% at t = 4, 22% at t = 2, and 22% at t = 0. The spontaneous from 10ME (B) (t equals the time of addition of cycloheximide) was 16% at t = 14, 20% at t = 12, 19% at t = 10, 19% at t = 8, 27% at t = 6, 24% at t = 4, 23% at t = 2, and 32% at t = 0.

of cycloheximide further demonstrates that these cells receive lytic signals from NK effectors but normally escape lysis by a counterlytic mechanism that requires protein synthesis (discussed below).

The fact that the NK-resistant wild-type B/C-N expresses an NK recognition determinant, which is indistinguishable from that expressed by the NK-sensitive variants 10ME and 10CR, implies that the transformation that results in the I phenotype does not involve the appearance of new determinants recognized by NK effectors. The transformation does, however, result in an increased sensitivity to the lytic mechanism initiated by NK cells. This is clearly different from the general findings with lymphoid targets where NK lysis is correlated with the presence of surface determinants on only NK-sensitive target cells (4, 14).

Because the I-type clone 10ME was derived from B/C-N in what appears to be a one-step mutation and selection for anchorage-independent growth (not NK sensitivity), we assume that anchorage-independent growth and sensitivity to NK-mediated lysis are related by a common gene control. Because there are also many examples where the same mutagenic and selection procedure yields anchorage independent (selected marker), NK-resistant (unselected marker) lines (C type) (1), there are many genes not common to the two. This can also be seen by considering the mutation(s) I to C within a lineage. These are correlated with a reduction in NK sensitivity yet the anchorage-independent phenotype is maintained (i.e., the cells do not revert to the N phenotype when they become NK resistant).

Although the precise nature of the selective pressure operating on the I type cells in ATxFL mice is not known, the tumor cell lines isolated from them (L51 and L53) show an increased tumorigenic potential when compared with their parental cell lines (10ME and 10CR). This increase in tumorigenicity could be the result of selection for either (a) NK resistance or (b) selection for increased tumorigenicity for some reason other than resistance to NK-mediated lysis.

If the selection pressure in ATxFL mice is low-level NK activity, then there is only one mutation (NK sensitive to NK resistant) required for an I-type cell to grow as a tumor in normal mice. However, we must assume that the direct selection for C-type variants from I-type lines does not occur in normal mice because their high level of NK activity results in the rejection of the I line before NK-resistant mutants can arise. In the ATxFL mouse, the NK activity is low enough to permit sufficient I-line growth for NK-resistant variants to arise; yet it is high enough to provide a selective enrichment for the NK-resistant variants.

If the selection pressure in ATxFL mice is for increased tumorigenic potential not related to NK activity, then a second mutation to NK resistance must be selected for in normal mice. The probability of obtaining C-type variants in I-cell populations by sequential selection, first in ATxFL then in normal mice, would be high. The probability of obtaining C-type variants by simultaneously selecting for both mutations in normal mice would be low. This would explain why C-type variants can only be selected sequentially. Our ongoing analysis of the tumorigenicity and NK sensitivity of cloned derivatives of the ATxFL passaged lines, L-51 and L-53, will resolve the number and nature of the mutations required for an I cell to express the C phenotype. Whatever the selective pressure is in ATxFL mice, the growth of the ATxFL-passaged cell lines, L51 and L53, as tumors in normal mice results in the selection of NK-resistant C-type cell lines (e.g., L88 and L81).

In spite of the good correlation between the tumorigenic potential of cells within a fibroblast lineage and NK sensitivity assayed *in vitro*, the observation that normal cells or C-type cells that have escaped tumor surveillance carry the NK recognition determinant and inhibit the killing of sensitive cells casts doubt on the role of NK as an effective *in vivo* surveillance mechanism. Because B/C-N and YAC-1 express the same recognition determinant, the *in vivo* efficiency of surveillance of transformed lymphoid cells by NK effectors must also be far from optimal. Without a special mechanism (e.g., the localization of NK effector cells at the tumor site or a high turnover rate for NK-target complexes), the competition by normal cells would be overwhelming because they are in vast excess over any *de novo* arising transformed cells. Even if a special mechanism exists, the competition by normal cells would still reduce the efficiency of tumor surveillance. The resolution of this apparent paradox is crucial to understanding the relationship between the NK system and cancer.

Two observations bear directly on the mechanism of NK lysis about which we will make only general comments. First, the rate of NK lysis depends upon the time when protein synthesis is inhibited during the NK assay. Second, NK-sensitive mutants, e.g., 10ME, can be derived from the NK-resistant B/C-N, and these cells show similar time-dependent patterns of lysis when protein synthesis is blocked.

The first point demonstrates that the observed rate of lysis is determined by two opposing processes, a lytic process and a counterlytic process. The fact that the observed rate of lysis increases if protein synthesis is blocked 2 h after initiation of the assay indicates that there is induction of a lytic mechanism within this time period. The activity of the lytic mechanism does not require protein synthesis because lysis proceeds when inhibitors of protein synthesis are added at the onset of the assay. The decline in total specific lysis as protein synthesis is inhibited at later times ($t > 2$ h) indicates that there is a constitutive counterlytic process the activity of which is dependent on protein synthesis. As a consequence, the addition of cycloheximide permits an assay of the total lytic activity because it effectively blocks counterlytic activity as well as further induction of the lytic mechanism without impairing the lytic activity. The reason for the decline in total specific lysis as cycloheximide is added at $t > 2$ h is simply that the time period decreases over which the total revealed lytic activity operates. As a first approximation, the induced lytic potential reaches a maximum in $t < 2$ h and stays at that level for the duration of the experiment.

The second point shows that the NK-sensitive mutant, 10ME, differs from the NK-resistant parent, B/C-N, in its constitutive level of counterlytic activity. The NK-sensitive mutant cannot differ from its NK-resistant parent either in the ability of NK effectors to recognize the two targets or in the capacity of the two targets to receive lytic signals and be induced to make the lytic mechanism; both targets are lysed similarly if protein synthesis is blocked at 2 h. This shows that in the absence of counterlysis, the induced lytic mechanism is roughly the same in both targets. In the presence of counterlysis, the B/C-N target is resistant (counterlysis is high), whereas the 10ME target is sensitive (counterlysis is low) because the mutation NK resistant to NK sensitive affects the constitutive level of counterlytic activity in the target.

Whereas the mutation NK resistant to NK sensitive shows that counterlysis is a property of the target, the question can be posed as to whether the induced lytic activity is synthesized by the effector or the target. Our unpublished data indicate that the induced lytic activity is synthesized by the target as a consequence of the NK

TABLE II
Comparison of the Characteristics of NK Activity Directed at Fibroblasts or Lymphoid Target Cells

	Lymphoid cells	Fibroblasts
NK-sensitive targets	Recognized by NK effectors and sensitive to the lytic mechanism	Recognized by NK effectors and sensitive to the lytic mechanism
NK-resistant targets	Not recognized by NK effectors (5) but presumed sensitive to the lytic mechanism	Recognized by NK effectors, but not sensitive to the lytic mechanism
Kinetics of lysis as measured by chromium release	Linear from $t = 0$	4-6 h lag then linear
Effect of cycloheximide on the lysis of NK-resistant targets	None*	Increases lysis
Number of NK recognition determinants (as determined by competition studies)	Several (5, 14)	Only one detected

* Data not presented.

effector delivering a lytic signal to the target. The experiments presented here, however, leave this question open.

Our results reveal several differences between the NK-lytic mechanism directed at fibroblasts and the lytic mechanism directed at lymphoid cells. Some of these differences are summarized in Table II. In spite of these differences the fact that YAC-1 competes for the lysis of 10ME, indicates the same NK effectors must recognize both 10ME (fibroblast) and YAC-1 (lymphoid) (see Fig. 9). Consequently the difference between the NK-mediated lysis of fibroblasts and lymphoid cells is probably not caused by different NK effector populations, one for fibroblasts and one for lymphoid cells. We are left then with two explanations for the differences summarized in Table II: (a) NK effector cells deliver one type of lytic signal, and fibroblastic targets react differently from lymphoid targets (most likely), or (b) NK effector cells are capable of delivering two types of lytic signals, one directed at fibroblast targets, the other directed at lymphoid targets.

One of the major differences between fibroblast and lymphoid targets is reflected in the kinetics of NK lysis. Fibroblast targets show a 4- to 6-h lag before lysis whereas lymphoid targets show no such lag (less than 0.5 h). Pretreatment of NK effectors by incubation with NK-sensitive targets does not shorten this lag when the assay is carried out on NK-sensitive targets (Fig. 11). Therefore, any putative activation of effector cells as a consequence of encountering sensitive targets cannot be the limiting factor responsible for the lag period. Further, the lag cannot be a result of the time it takes to induce a sufficient level of autolytic enzyme in the target because the level is maximal in about 2 h (see Fig. 12). At the moment the lag can be accounted for as the time required to activate this autolytic enzyme in the target and/or release the ^{51}Cr . Lymphoid targets must differ in one or both of these properties.

Our studies show that if NK activity plays an important role in tumor surveillance, there is a major missing piece as to how it can function efficiently in vivo.

Summary

Detailed analysis of the natural killer (NK) activity directed at nontumorigenic cell lines and their transformed tumorigenic derivatives has revealed a paradox.

On the one hand, a correlation has been found between the tumorigenic potential of chemically transformed fibroblast cell lines and their sensitivity to NK cells *in vitro*. Nontransformed cells (N-type cell lines) and cells tumorigenic in normal mice (C-type cell lines) are resistant to NK-mediated lysis. In contrast, cell lines that are tumorigenic in ATxFL mice (these mice are very low in NK activity), but not in normal mice (I-type cell lines) are sensitive to NK-mediated lysis. These findings support the concept that NK activity is involved in host surveillance against tumors.

On the other hand, NK-resistant fibroblasts, whether taken directly from animals or derived as tumorigenic or nontumorigenic cell lines, compete with NK-sensitive target cells to inhibit their lysis by NK effectors. Not only are both NK-sensitive and -resistant cells recognized by NK effectors but both receive lytic signals from NK effector cells. Target cell resistance is a result of a protein synthesis-dependent mechanism that prevents lysis such that in the presence of inhibitors of protein synthesis all fibroblasts tested are NK sensitive. Those fibroblasts that are normally sensitive to NK-mediated lysis must be deficient in their ability to produce or respond to this counterlytic mechanism. These findings are in contrast with the general findings when lymphoid cells are studied as NK targets where sensitivity appears to be a result of recognition by NK effectors.

Because our findings show that transformed and normal cells express the same recognition determinants, in order for NK activity to play an important *in vivo* role in tumor surveillance, a mechanism must operate to permit NK effectors to find their targets *in vivo*. In the absence of a special discrimination mechanism, the killing of NK-sensitive transformants that arise autochronously would be less than optimal as a consequence of competition by the normal, NK-resistant, cells.

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